SPECIFICATION

γδT CELL IMMUNOACTIVITY ENHANCERS CONTAINING EXTRACT OF LENTINUS EDODES MYCELIUM

FIELD OF THE INVENTION

The present invention relates to the development and preparation of γδT cell activity enhancers and therefore immunopotentiators containing an extract of Lentinus edodes mycelium.

The present invention also relates to the development and preparation of foods, drinks and feeds containing an extract of Lentinus edodes mycelium and having a γδT cell activity-enhancing effect and therefore an immunopotentiating effect.

The present invention also relates to the development and preparation of antitumor agents, therapeutic agents against bacterial infections and therapeutic agents against viral infections containing an extract of Lentinus edodes mycelium.

PRIOR ART

Characteristics of γδT cells

Peripheral T cells in animal blood are mainly classified into two groups based on the type of their cell surface antigens referred to as T cell receptors (TCR). One type is an αβT cell bearing TCR α and β chains on their cell surfaces, and the other is a γδT cell bearing TCR γ and δ chains. γδT cells are cytotoxic killer cells present at a level of only a few to about 10% in normal peripheral blood

and lymphoid tissue in humans, and the characteristics of which are quite distinct from those of αβT cells.

In humans, γδT cells are present in the intestinal tract, skin and peripheral blood or the like and elicit a local immunity. So far, γδT cells have been reported to have functions such as cytotoxicity against cancer cells, and protective activity against bacterial or virus infections, etc.

<u>Phylaxis activity of γδT cells</u>

Some γδT cells in the spleen and other organs produce cytokines such as IL-4 or IFN-α in response to infection. It has been shown under experimental conditions that when these cells are lacking, resistance to bacterial infections is reduced. For example, there is one report which describes that resistance to Mycobacterium tuberculosis infection decreased in mice treated with a γδ-type TCR antibody to transiently inhibit functions of γδT cells or mice deficient in the TCR γ gene (Ladel C. et al., Eur. J. Immunol., 1995, 25:2877-2881). Another report describes that γδT cells appear during the early stage of infection with Listeria monocytogenes (Hiromatsu K. et al., J. Exp. Med., 1992, 175:49-56). These findings suggest that γδT cells play an important role in protecting against bacterial infections.

It has also been reported that chronic hepatitis B virus infection induces the growth of γδT cells in the liver and spleen (Ozaki S. et al., J. Med. Invest., 1998,

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44:215-217); and that vaccinia virus markedly increases during the early stage of infection in γδT cell-deficient mice as compared with normal mice (Welsh RM, et al., Immunol. Rev., 1997, 159:79-93). These findings suggest that γδT cells act not only on bacterial infections but also on viral infections.

Cytotoxicity of γδT cells against cancer cells

γδT cells are a class of T cells that are capable of specifically targeting and killing autologous cancer cells, but show no cytotoxicity to autologous normal lymphocytes (such as αβT cells). In this respect, in cancer therapy which employs activated γδT cells there is very little danger of side effects. In contrast to γδT cells, αβT cells are known to kill autologous leukocytes rather than autologous cancer cells, and thus in cancer therapy which employs activated αβT cells there is a likelihood that serious side effects will occur. In view of this, cancer therapy which employs activated γδT cells is desirable.

Moreover, γδT cells have similar characteristics to NK cells such as their MHC-nonrestricted cytotoxicity against cancer cells. γδT cells are present in peripheral blood of children at about 10% but decrease with age. This suggests that the increase in the occurrence of cancer with age may be related to a decrease in γδT cells. In peripheral blood of chicken, sheep, cow or the like, γδT cells are found at levels as high as 15-50%. The low incidence of tumors in these animals suggests that the

presence of peripheral γδT cells may contribute greatly to the inhibition of cancer.

Pharmacological effects of Lentinus edodes

Lentinus edodes (Shiitake) is a common edible mushroom found in both Japan and China, and has been cultivated in Japan for around 300 years. The part of the mushroom used as food econsists of the reproductive body, also referred to as the fruiting body of fungi, which produces sporesfor reproduction, while the vegetative body includes hyphae which produce mycelia extending into a growing area such soil or logs.

Lentinus edodes has long been believed to have some effect against a variety of diseases and symptoms, but it is only relatively recently that any pharmacological effect has been elucidated. Various effects of extract of Lentinus edodes mycelium are reported, these include the inhibition of oncogenesis in the large bowel and liver and the growth of transplanted tumor cells and increased survival of animals in carcinogenesis experiments in rats and mice (N. Sugano et al., Cancer Letter, 27:1, 1985; Y. Suzuki et al., Journal of the Japan Society of Coloproctology, 43:178, 1990, etc.); mitogenic activity (T. Tabata et al., Immunopharmacology, 24:57, 1992; Y. Hibino et al., Immunopharmacology, 28:77, 1994, etc.); enhanced antibody production and inhibitory effects against immunological hepatocyte damage caused by ADCC (antibody-dependent cell-mediated cytotoxicity) (Y. Mizoguchi et al., Journal of Hepato-Biliary-Pancreatic Study, 15:127, 1987).

These findings have acted as a catalyst for concentrated research into the pharmacological effects of ingredients of Lentinus edodes in the medical and pharmaceutical field. As a result, researchers have discovered that some of the constituents of Lentinus edodes can be used in the treatment of cancer or other diseases by restoring immune function in humans, and further that such constituents may also inhibit the onset of cancer.

In accomplishing the present invention the inventors have aimed to further elucidate pharmacological effects of an extract of Lentinus edodes mycelium, and also to search for new applications of the extract in the form of drugs, foods, drinks, feeds, etc.

One object of the present invention is to develop and provide a γδT cell activity enhancer and therefore an immunopotentiator such as an antitumor agent, therapeutic agent against bacterial infections and therapeutic agent against viral infections containing an extract of Lentinus edodes mycelium.

Another object of the present invention is to use a γδT cell activity enhancer containing an extract of Lentinus edodes mycelium or an immunopotentiator containing an extract of Lentinus edodes mycelium to treat a tumor in a subject.

DISCLOSURE OF THE INVENTION

As a result of intensive studies aimed at solving the above problems, the present invention has been accomplished

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on the basis of the finding that an extract of Lentinus edodes mycelium exhibits an effect of remarkably enhancing the activity of γδT cells.

Accordingly, the present invention has been developed and provided γδT cell activity enhancers and therefore immunopotentiators such as antitumor agents or therapeutic agents against bacterial or viral infections containing the extract of Lentinus edodes mycelium.

The present invention also provides methods developed for treating tumor, bacterial infections and viral infections using the extract of Lentinus edodes mycelium.

γδT cell activity enhancers or immunopotentiators of the present invention may be in the form of a pharmaceutical composition containing the extract of Lentinus edodes mycelium and optionally a pharmaceutically acceptable carrier.

γδT cell activity enhancers or immunopotentiators of the present invention may be administered in the form of injection or oral, mucosal, gastrointestinal or percutaneous formulation.

γδT cell activity enhancers or immunopotentiators of the present invention may also be in the form of a food, drink or feed.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows that the proportion of γδT cells in peripheral blood increases following administration of the extract of Lentinus edodes mycelium of the present

invention.

FIG. 2 shows the results of flow cytometric analysis of γδT cells before and after administration of the extract of Lentinus edodes mycelium.

FIG. 3 shows that the proportion of αβT cells in peripheral blood decreases following administration of the extract of Lentinus edodes mycelium of the present invention.

THE MOST PREFERRED EMBODIMENTS OF THE INVENTION

An extract of Lentinus edodes mycelium used for enhancing the activity of γδT cells according to the present invention refers to an extract obtained by crushing and decomposing mycelia grown from Lentinus edodes cultured on a solid medium, or a solid medium itself containing Lentinus edodes mycelia in the presence of water and an enzyme.

An extract of Lentinus edodes mycelium used herein is preferably obtained by, but not limited to, the following process. Lentinus edodes spawn is inoculated on a solid medium based on bagasse (sugar cane residue) and defatted rice bran to grow mycelia, and then the solid medium containing the grown mycelia is delignified so that 30% by weight or less is able to pass through a 12-mesh sieve. To this delignified solid medium are added water and one or more enzymes selected from cellulase, protease or glucosidase while maintaining said solid medium at a temperature of 30-55°C, and said solid medium is crushed

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and ground in the presence of said enzyme so that at least 70% by weight of bagasse fiber is able to pass through a 12-mesh sieve. Then, the temperature is raised to 95°C to ensure inactivation of the enzyme and sterilization, and the resulting suspension is filtered to give an extract of Lentinus edodes mycelium. The extract of Lentinus edodes mycelium as prepared above may be directly used in γδT cell activity enhancers of the present invention, but conveniently concentrated and freeze-dried into powder to be stored and used in various forms. The freeze-dried product is a brown powder with hygroscopic characteristics and has a peculiar taste and odor.

The extract of Lentinus edodes mycelium was tested for its in vivo γδT cell activity-enhancing effect by the method described in the examples below, and it will be seen from these examples that it has a remarkable in vivo γδT cell activity-enhancing effect.

γδT cell activity enhancers of the present invention are effective for treating and/or preventing tumor induced by tumor cells to which γδT cells are cytotoxic. γδT cell activity enhancers of the present invention are characterized in that they enhance the activity of γδT cells and thereby lead to the destruction of tumor cells under the action of the activated γδT cells, rather than having a direct action on specific tumor cells. Consequently, tumor cells to be treated with γδT cell activity enhancers of the present invention may be not only malignant tumor cells but also benign tumor cells, and are

not limited to specific tumor cells. In addition, γδT cell activity enhancers containing the extract of Lentinus edodes mycelium and optionally a pharmaceutically acceptable carrier can be used in the form of both therapeutic and prophylactic compositions.

γδT cell activity enhancers of the present invention can also be used as therapeutic and/or prophylactic compositions for bacterial or viral infections. γδT cell activity enhancers of the present invention are intended to enhance the activity of γδT cells and thereby to remove infecting bacteria or viruses from the patient rather than to directly act on specific bacteria or viruses. Bacterial or viral diseases that can be treated with γδT cell activity enhancers of the present invention include such diseases as , but are not limited to, those caused by Mycobacterium spp. Listeria monocytogenes, hepatitis viruses (types A, B and C), human immunodeficiency virus, vaccinia virus and the like.

γδT cell activity enhancers of the present invention in the form of a therapeutic and/or prophylactic composition are administered most preferably via the oral route, but may also be administered via intravenous, intraperitoneal, subcutaneous, intramuscular, nasal, percutaneous or other route. Dosage forms suitable for oral administration include, but are not limited to, tablets, capsules, powders, granules, solutions, syrups, etc. Dosage forms suitable for nasal or percutaneous administration include, but not limited to, cataplasms,

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patches, etc.

Pharmaceutically acceptable carriers include, but are not limited to, suitable excipients, binders, disintegrators, lubricants, flavoring agents, colorants, solubilizers, suspending agents, coating agents or the like known in the art.

Pharmaceutically acceptable carriers that can be optionally mixed in γδT cell activity-enhancing formulations of the present invention include, but are not limited to excipients such as lactose, dextrose, starch, crystalline cellulose; binders such as starch, gelatin, methyl cellulose, polyvinylpyrrolidone; disintegrators such as starch, calcium carboxymethylcellulose, carboxymethyl starch; lubricants such as talc, stearates; coating agents such as sucrose, talc, gelatin; as well as various brighteners, flavoring agents, colorants, corrigents, solubilizers, stabilizers, suspending agents, absorbefacients or the like known in the art depending on the purpose. For use as injections, various diluents commonly used in this field of art such as water or ethyl alcohol can be used.

The dose of γδT cell activity enhancers of the present invention is determined by physicians taking into account the age, weight and condition of the subject, the route of administration and other factors. The dose is not strictly limited because the extract of Lentinus edodes mycelium contained in γδT cell activity enhancers of the present invention is highly safe, and has been

traditionally ingested as a component of food. For example, the extract of Lentinus edodes mycelium is normally administered preferably at a dose of 100 mg - 10000 mg several times (about 2-3 times) daily (a total of 200 mg - 30000 mg daily), more preferably 500 mg - 5000 mg three times daily (a total of 1500 mg - 15000 mg daily), most preferably 1000 mg - 1500 mg three times daily (a total of 3000 mg - 4500 mg daily). It may be administered in combination with other antitumor agents.

γδT cell activity enhancers of the present invention can be provided in a dosage form also suitable for adoptive immunotherapy for treating tumor. Adoptive immunotherapy refers to a type of anti-tumor therapy intended to kill tumor cells by transferring into a subject sensitized cells, normally lymphocytes. In the case of the present invention, γδT cells are initially isolated from peripheral blood of the subject, and the isolated γδT cells are activated in vitro by a γδT cell activity enhancer of the present invention, and then the activated γδT cells are returned into the subject. As a result, tumor cells in the subject can be destroyed by the action of the activated γδT cells.

γδT cell activity enhancers of the present invention may be the extract of Lentinus edodes mycelium itself or pharmaceutical or veterinary compositions comprising a γδT cell activity enhancer containing the extract of Lentinus edodes mycelium and a pharmaceutically acceptable carrier.

γδT cell activity enhancers of the present invention can also be provided in the form of a food. Preferred

forms of food include powders, granules, pastes, jellies, etc. Granules desirably are supplemented with sugars such as lactose to add a sweet taste. γδT cell activity enhancers of the present invention can also be provided in the form of a drink. These foods or drinks may be supplemented with vitamins, minerals such as calcium, alcohols, deodorants such as polyphenols in addition to the extract of Lentinus edodes mycelium. These foods or drinks include the categories of specific health foods, medical foods or the like.

γδT cell activity enhancers of the present invention can also be provided in the form of a feed or feed additive. γδT cell activity enhancers of the present invention can be used as a feed or feed additive for domestic animals to treat and/or prevent tumor occurring in domestic animals or to treat and/or prevent bacterial or viral infections in domestic animals. As a result, the amount of therapeutic agents such as antibiotics currently used can be reduced, thereby reducing farming costs. Another advantage is that the period during which shipment of animals is suspended due to the administration of antibiotics can be shortened.

In vivo γδT cell activity-enhancing effect was tested in human subjects as follows. Human subjects initially received 3.6 g of the extract of Lentinus edodes mycelium bulk powder daily for 7 days (a total of 25.2 g). Then, the proportion of γδT cells in peripheral blood after administration of the extract of Lentinus edodes mycelium was determined by flow cytometry as compared with the

proportion before administration.

The following examples, which further illustrate the present invention, should not be taken as limiting the the scope of the invention thereto. Various changes and modifications can be made by those skilled in the art and such changes and modifications are also included in the scope of the present invention.

EXAMPLES

Example 1: Preparation of an extract of Lentinus edodes mycelium

A solid medium consisting of 90 parts by weight of bagasse and 10 parts by weight of rice bran was soaked with an appropriate amount of pure water, and then inoculated with Lentinus edodes spawn and allowed to stand in an incubator at controlled temperature and humidity to grow mycelia. After mycelia spread over the solid medium, the bagasse base was delignified so that 24% by weight or less may pass through a 12-mesh sieve. To 1.0 kg of this delignified medium were added 3.5 L of pure water and 2.0 g of purified cellulase while maintaining the solid medium at 40°C to prepare a medium-containing mixture.

Then, the medium-containing mixture was circulated by a variable speed gear pump, during which the solid medium was crushed and ground under the gears for about 200 minutes so that about 80% by weight of bagasse fiber may pass through a 12-mesh sieve. The medium-containing mixture was crushed and ground while the temperature of

said mixture was gradually increased. Then, the medium-containing mixture was further heated to 90°C and allowed to stand for 30 minutes. This heating to 90°C ensured inactivation of the enzyme and sterilization. The resulting medium-containing mixture was filtered through a 60-mesh filter cloth to give an extract of Lentinus edodes mycelium solution, which was concentrated and then freeze-dried into an extract of Lentinus edodes mycelium bulk powder.

The extract of Lentinus edodes mycelium as prepared above contained 25.3% (w/w) carbohydrates determined by the phenol-sulfuric acid method, 19.7% (w/w) proteins determined by the Lowry method and 2.6% (w/w) polyphenols determined by the Folin-Denis method using gallic acid as standard. The extract of Lentinus edodes mycelium further contains 8% crude fat, 22% crude ash and about 20% soluble nitrogen-free materials other than carbohydrates.

The extract of Lentinus edodes mycelium had a sugar composition (%) as follows: Xyl 15.2, Ara 8.2, Man 8.4, Gul 39.4, Gal 5.4, GlcN 12.0, GluUA 11.3.

Example 2: In vivo γδT cell activity-enhancing test of the extract of Lentinus edodes mycelium

Three human subjects (subjects A-C) received orally 3.6 g/day of the extract of Lentinus edodes mycelium bulk powder daily for 7 days (a total of 25.2 g). After the period of administration of the extract of Lentinus edodes mycelium, peripheral blood was collected from the human

subjects. The proportion of γδT cells in peripheral blood collected after administration was determined by flow cytometry as compared with the proportion in peripheral blood collected before administration. The results are shown in Figs. 1 and 2.

In all of the three subjects, the proportion of γδT cells in peripheral blood increased by an average of 40% or more after administration of the extract of Lentinus edodes mycelium as compared with before administration.

Table 1: Increase of the proportion of γδT cells in peripheral blood after administration

	Subject A	Subject B	Subject C	Mean ± SEM
Increase	124.39%	146.15%	150.00%	140.18%±7.97%

Peripheral blood collected from the same subject before and after administration was tested for other markers than γδT cells. As a result, the extract of Lentinus edodes mycelium showed no proliferative activity on αβT cells and on average the proportion of αβT cells decreased (Fig. 3).

<u>INDUSTRIAL APPLICABILITY</u>

γδT cell activity enhancers containing the extract of Lentinus edodes mycelium of the present invention were found to actually activate γδT cells. Thus, γδT cell activity enhancers of the present invention can be used for preventing or treating tumor, bacterial infections and

viral infections because they have the effect of protecting living bodies against tumor, bacterial infections and viral infections by inducing cytotoxic activity against tumor cells, antibacterial activity and antiviral activity of γδT cells. Moreover, γδT cell activity enhancers of the present invention are suitable for wide industrial application because they can be used safely without side effects.

They can also be used in domestic animals with bacterial and/or viral infections to reduce the amount of therapeutic agents currently used such as antibiotics, thereby reducing costs for raising. They also have the advantage that the period during which shipment is suspended can be shortened because antibiotics are not used.